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F20C, A NEW FLUORESCENT MEMBRANE PROBE, MOVES MORE SLOWLY IN MALIGNANT AND MITOGEN-TRANSFORMED CELL MEMBRANES THAN IN NORMAL CELL MEMBRANES

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Summary

New fluorescent probes of membrane mobility can be introduced into cell membranes at single points with particles of a membrane mobility agent, A_2C. The initial entry of fluorescence from the particle into the cell membrane and the subsequent lateral spread of fluorescence have been observed for cells in suspension. A dramatic difference between the behavior of normal lymphocytes and that of mitogen-transformed and mastocytoma cells is found. Both the initial entry and the spreading of fluorescence are much slower in the transformed and tumor cells than in the normal cells at 18°C. Entry and spread of fluorescence in normal cells become slow enough to be observed only at 12°C or below.

Introduction

The membrane mobility agent A_2C (see formula) [1] promotes cell fusion [2] as well as the lateral mobility of wheat germ agglutinin · membrane component complexes in mastocytoma cell membranes (i.e., cap formation at the expense of agglutination) [3]. In order to study the interaction of the mobility agents with the cell membrane we have recently developed new fluorescent membrane probes, as fluorescent probes of mobility in membranes (formula below). The use of probe-loaded mobility agent allows a direct view of the entry and spread of the mobility agent into cell membranes [4].

We have utilized F20C as a probe for comparing the behavior of normal, mitogen-transformed and malignant cell membranes and can now report that there is a substantial difference at certain temperatures in the speed with which

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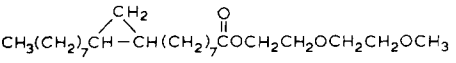
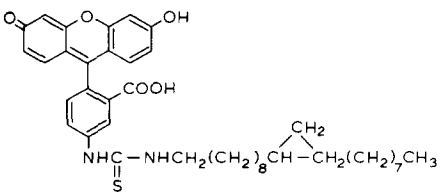


A_2C



F20C

the probe spreads through the cell membranes, the spread being much faster in the normal cell than in malignant or mitogen-transformed cell membranes at 18°C. Only upon lowering the temperature from 18°C to below 12°C did a decrease in the lateral diffusion rate of fluorescent probe in normal cell membranes become apparent.

Thus, membrane component mobility and intrinsic fluidity may not be greater for malignant cells than for normal cells [5—9].

Materials and Methods

The cells used included: (a) Thymus-derived lymphocytes of normal 4—6-week-old C57 B1/6 mice. (b) Mitogen-treated thymocytes (hereafter referred to as transformed) and untreated thymocytes: thymus-derived lymphocytes were placed in tissue culture medium RPMI-1640, supplemented with 5% horse serum and 10^{-5} M 2-mercaptoethanol with and without staphylococcal enterotoxin B (SEB, 10 μg/ml) as transforming agent [10]. Cells were used after 72 h in tissue culture, at which time the sample treated with SEB contained approx. 60—75% blasts. (c) Mastocytoma cells (line P-815-X2) grown in Dulbecco's modified Eagle medium, supplemented with 10% horse serum [11]. Cells were used at the end of the log-phase growth (beginning of stationary phase), usually at approx. 72 h after transferring 10^5 cells/ml to fresh medium. Cells were washed in phosphate-buffered saline and resuspended to a concentration of $4—6 \cdot 10^6$ cells/ml.

F20C was synthesized by the general procedure of Coons and Kaplan [37] as modified by Steinbach [38] from 5-isothiocyanatofluorescein and the C_{20}-amine derived from methyl dihydrosterculate by straightforward organic procedures (Kosower, E.M. and Iny, G., unpublished results).

A_2C (available from Makor Ltd., P.O. Box 6570, Jerusalem, Israel), was added to a methanol solution of F20C, the methanol evaporated by a stream of nitrogen, and the residue dispersed in saline by sonication for 15—30 s. The dispersion was then added to the cell suspensions (usually in equal volumes) and the mixture maintained at various temperatures. Aliquots were removed at intervals for viewing and photography at appropriate temperatures using a Zeiss microscope equipped with a high-intensity mercury lamp for incident light excitation of fluorescence.

Results and Discussion

Suspensions of A_2C-F20C, prepared as described above contain fluorescent particles with sizes between 0.4 and 1.6 μm in diameter. The interaction of

the particles with cell membranes comprises of four observable steps: (1) approach of the particles to the cell, (2) contact and sticking of the particle to the cell, (3) local entry of fluorescence from the particle into the cell membrane, and (4) lateral spread of fluorescent compound throughout the cell membrane. (A more detailed analysis of the steps involved in the interaction of membrane mobility agent particles with cells is discussed elsewhere [39].) The length of time required for the above steps is dependent upon cell type and temperature. Our results for a number of cell types are illustrated in Figs. 1—3.

For all of the cell types, the time for the overall process (the time from the addition of reagent to the cell suspension until fluorescence has spread throughout the whole of the cell membrane) at 37°C is between 60 and 120 s, with much of that period required for the approach of the particle to the cell. The following steps, sticking, initial entry and lateral diffusion through the

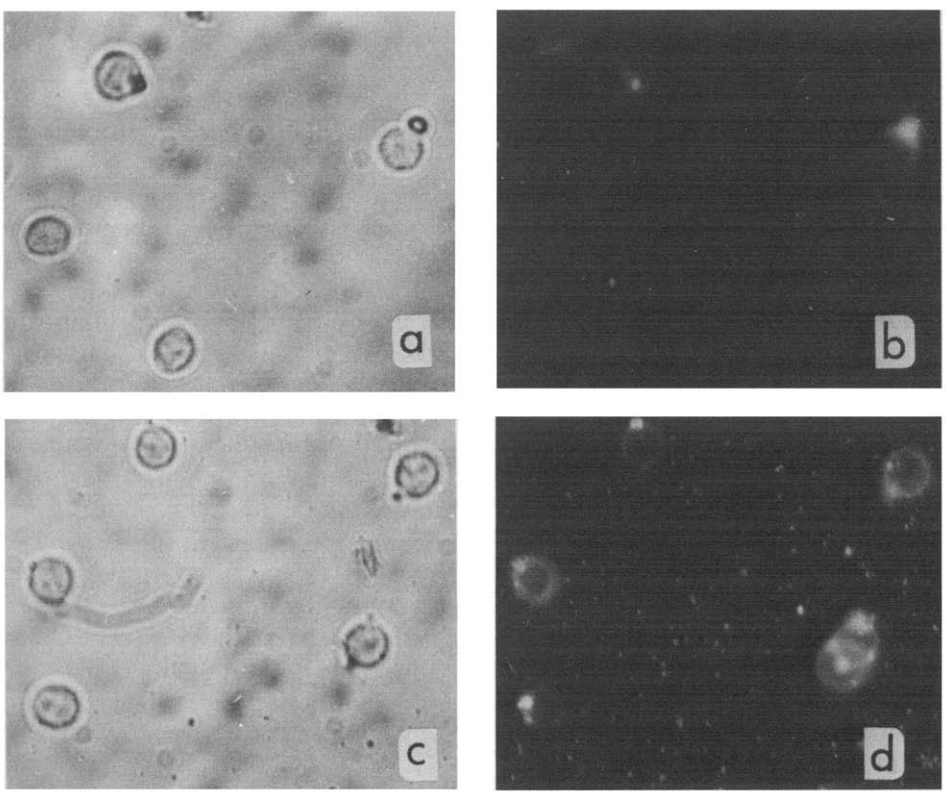


Fig. 1. Normal mouse thymocytes treated with A_2C-F20C. Lymphocyte suspensions ($4 \cdot 10^6$ cells/ml buffer) were mixed with an equal volume of an A_2C-F20C suspension (the A_2C-F20C suspension contained 1.2 μmol A_2C and 1.25 nmol F20C/ml isotonic saline. For details of preparation of suspension and of cells see Materials and Methods). The combined suspension was placed at 37°C without shaking. a,b: Fields were photographed 60 s after mixing, first to exhibit fluorescence, and then under ordinary illumination. Two fully fluorescent cells are seen along with two dark (non-fluorescent) cells. A number of fluorescent particles are visible in the medium. The fluorescent cells carry fluorescent particles. c,d: Fields were photographed 240 s after mixing, first for fluorescence, and then under ordinary illumination. All cells are fully fluorescent and carry fluorescent particles. Many fluorescent particles are visible in this field.

whole membrane, transpire within 1 s or less. Hardly a cell is seen to which fluorescent particles are stuck without that cell being fluorescent; at any given time, the cells under observation are either not fluorescent or are completely fluorescent. (Fig. 1). In a few cases, we observed the approach of a fluorescent particle to a 'dark' cell, contact, and then a burst of fluorescence which 'instantaneously' engulfed the whole membrane.

Approach and sticking of particles to all cells are somewhat slower at temperatures lower than 37°C. At 16—18°C, it takes about 2—3 min before appreciable numbers of particles are found in contact with cells, while at 10—12°C, 3—5 minutes are needed for the same distribution to be observed.

For normal mouse thymus derived lymphocytes, at 18°C some cells could be seen with fluorescent particles attached to them for about 1 min without the cells being fluorescent, so that the steps of sticking and entry are slower at 18°C than at 25°C. Once entry had occurred, the fluorescence spread through the cell at 18°C was fast and not distinguishable from that seen at 25°C or 37°C. "Bursts" of fluorescence, when seen, spread throughout the membrane within 1 s or less. At lower temperatures (10—12°C), dark cells carrying fluorescent particles could be seen for several minutes before the fluorescence spread through the membrane, a spread which was slow in comparison with that seen at higher temperatures, and requiring up to 60 s for completion, too rapid for photography of partially fluorescent lymphocytes. Only if the lymphocytes were maintained at a temperature of 5—6°C was the spread of fluorescence slow enough (approx. 300 s for completion) to make photography possible. If the lymphocytes were kept at 1—2°C, fluorescent particles were seen attached to cells but neither spreading nor local membrane fluorescence was noted. Thus, an observable change in the rate of spread of the fluorescence probe occurs at a temperature near 12°C for the normal lymphocyte, but the process is still fast at 5°C.

Mitogen-transformed lymphocytes behaved rather differently from normal cells at 18°C. Particles stuck to cells could easily be observed for several minutes. More important, fluorescence spread through the membrane of the transformed cell was much slower than seen in the normal cells, requiring approx. 600 s for completion. Samples viewed during that time revealed partially fluorescent cells as illustrated in Fig. 2. Non-transformed thymocytes, after 72 h in tissue culture, behaved like freshly isolated cells, exhibiting rapid fluorescence spread at 18°C.

In the case of malignant mastocytoma cells treated at 21 or 24°C, the time required for local entry was slightly longer than that needed at 37°C (approx. 90—120 s), but the spread of fluorescence throughout the cell membrane was not distinguishable from that seen at 37°C (approx. 1 s). At 18°C, however, the malignant mastocytoma cells behaved like the transformed thymocytes: following attachment of the fluorescent particles to cell membranes, the bright particles could be seen stuck to 'dark' cells for 300—400 s before the entry of fluorescence into the cell membrane. Once lateral diffusion had begun, it required approx. 600 s for completion so that partially fluorescent cells could be photographed. A series showing the gradual spread of fluorescence through the mastocytoma cell membrane is given in Fig. 3. At 15°C, fluorescent particles can be seen on the dark mastocytoma cells for approx. 600 s;

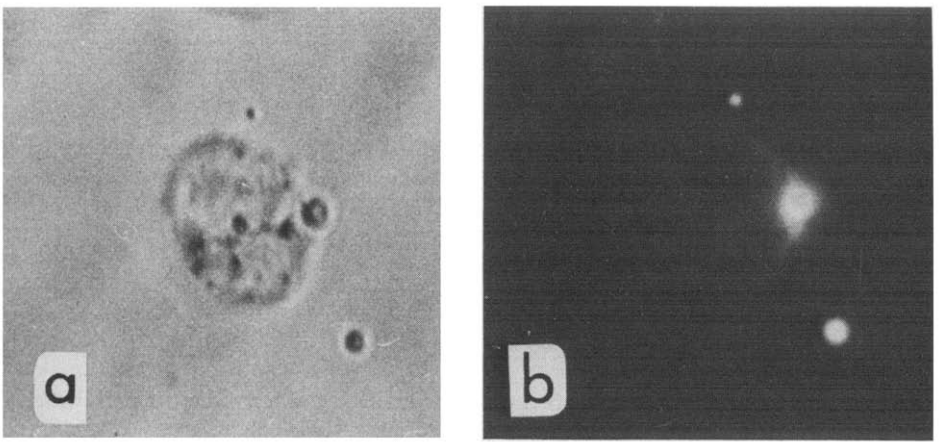


Fig. 2. Transformed mouse thymus-derived lymphocytes treated with A_2C-F20C suspension. (See legend to Fig. 1 for quantities, Materials and Methods for transformation of cells and details of preparation). Temperature: 18°C. Approx. 600 s after mixing, a cell was photographed under fluorescence exciting light and then under ordinary illumination. A fluorescent particle is seen attached to the blast cell and the membrane is partially fluorescent. Two fluorescent particles are also visible in the medium.

subsequently, the initial stages of entry of fluorescence could be seen over a period of approx. 200 s and lateral diffusion throughout the cell membrane required approx. 1200 s for completion. Cells maintained at lower temperatures (11—12°C) showed attached particles but no local entry or spread of fluorescence were seen. In addition, the particles were not firmly attached to the cells as manifested by apparent motion of the particles or loss of the particles from cells with which they had been associated, a loss not seen at higher temperatures. Cells maintained at 11—12°C or at lower temperatures could be warmed to 37°C after contact between particles and cells had been established with a resultant instantaneous spread of fluorescence throughout the cell membrane, similar to that seen in cells for which the whole procedure has been carried through at 37°C. Thus, for malignant mastocytoma cells, a sharp change in the rate of entry and lateral spread of a fluorescent probe through the cell membrane occurs at a temperature between 18 and 21°C.

Pre-incubation of the cells with 10 mM sodium azide had no effect on any of the subsequent behavior observed after the addition of A_2C-F20C. The time for the spread of fluorescence at various temperatures is summarized in Fig. 4.

Our experiments show how readily the entry of an agent into the cell membrane can be seen with the aid of probe-loaded membrane mobility agent. The fluorescence is not seen inside the cells under the conditions we have used unless the cell is injured, in which case a very bright total fluorescence of the cell is observed.

The most probable mechanism for the entry of the particle contents into the cell membrane is via fusion of the particle with the membrane [39]. In human red cells, A_2C treatment leads to cup formation (Kosower, N.S., Kosower, E.M., Wegman, P. and Zipser, J., unpublished results). Sheetz and Singer have proposed [12] that reagents which give rise to cup formation in erythrocytes are distributed within the bilayer membrane in an asymmetric fashion with

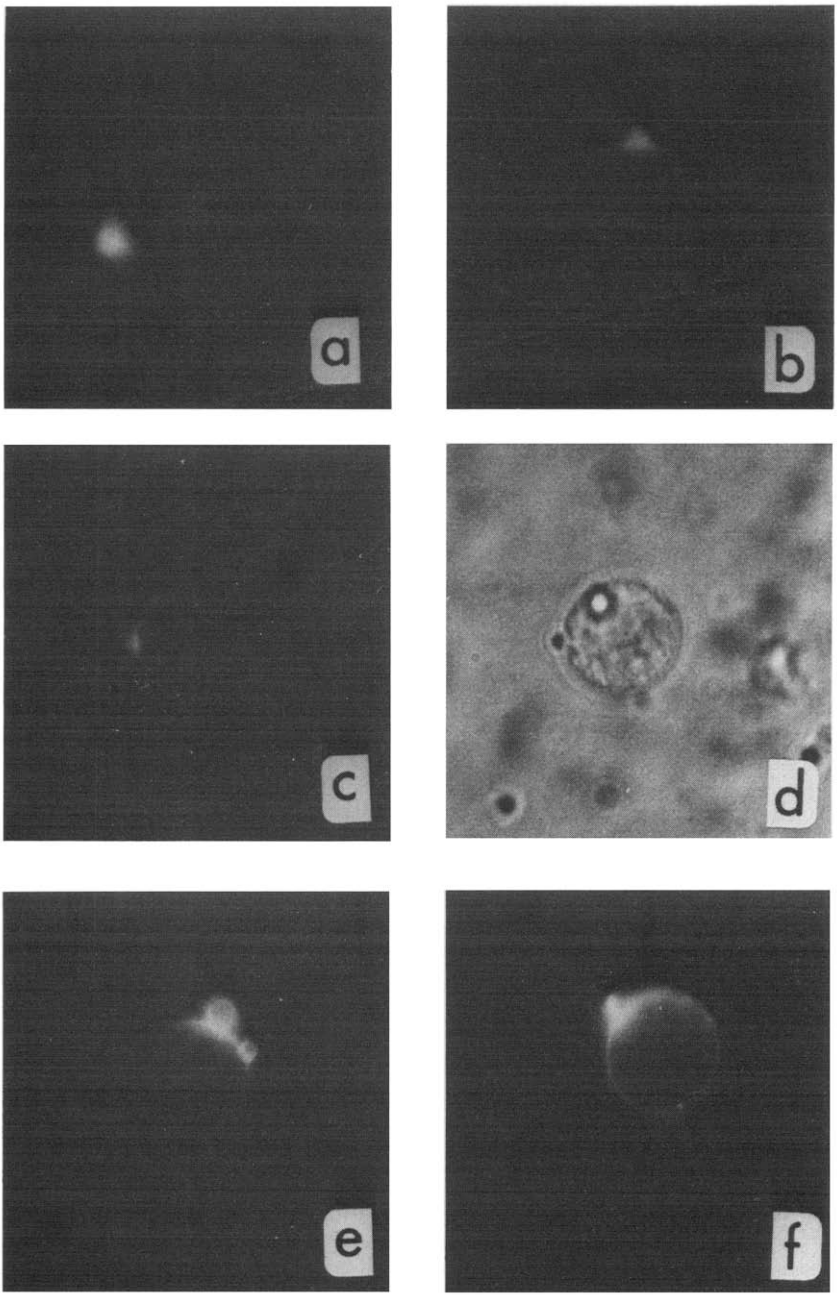


Fig. 3. Mastocytoma cells mixed with A_2C-F20C suspension at 18°C. (See legend to Fig. 1 for quantities, and for details on cells and on preparation of suspension, see Materials and Method). a: Approximately 300 s after mixing. A fluorescent particle is attached to a cell, but little or no fluorescence has entered the cell membrane. b: Approximately 460 s after mixing. A fluorescent particle is attached to a cell membrane and fluorescence has spread for a small distance into the membrane. c,d: Approximately 600 s after mixing. A fluorescent particle is attached to the cell membrane, and fluorescence has spread around about one-third of the membrane. e: Approximately 600 s after mixing. Two fluorescent particles are attached to a cell membrane and fluorescence has spread through part of the cell membrane. f: Approximately 960 s after mixing. A particle is attached to the cell. The whole cell membrane is fluorescent.

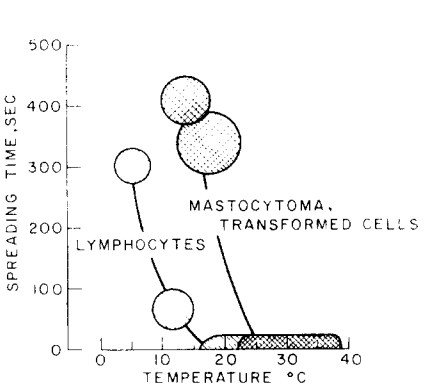


Fig. 4. Spread of fluorescence in membranes of normal lymphocytes, transformed lymphocytes and mastocytoma cells. The spreading time for fluorescence at different temperatures in various cell types is summarized in this figure. The variation in observed spreading times is indicated by the marked areas.

preference for the inner portion of the bilayer. One might then suggest that A_2C is initially delivered to the membrane bilayer with some preference for the inner portion, but the distribution of F20C is less certain and awaits further investigation.

The present experiments demonstrate a dramatic difference between the behavior of normal lymphocytes on the one hand and that of mitogen-transformed thymocytes and the malignant mastocytoma cells on the other, with respect to the entry and lateral spread of a lipid-like reagent. Normal thymocytes exhibit a rapid lateral spread of F20C within the membrane at temperatures above 12°C, 1 s or less being the time needed for the membranes to become fully fluorescent. Only at 12°C or lower is the spread of fluorescence slow enough to be observed. Transformed thymocytes and mastocytoma cells, in contrast, show neither entry nor spread of fluorescence at 12°C or below, while at 18°C, the lateral spread is slow enough to be observed and photographed. In fact, the lateral spread at 18°C in transformed and malignant cells of the types we have used is slower than the lateral spread of fluorescence within normal thymocyte membranes at 6°C. The difference in the observed times for the two classes of cell membranes may be as much as a factor of 1000 at 18°C.

Since the difference between the two classes is apparently small at 25 or 37°C, we must conclude that a high temperature factor for lateral diffusion through membranes is operative, and is more evident in the case of the transformed and malignant cell membranes. (Rates of spin-labeled phospholipid diffusion through cell membranes or lipid bilayers [13,14] imply that the time for small molecule spread through a cell membrane would be of the order of 1 s at 37°C). Since such a high temperature factor would not be expected for diffusion through a purely lipid bilayer over the range from 18—25°C, we may infer that other membrane constituents can exert a strong influence on the entry from particles into the membrane and the lateral spread through the membrane of lipid-like molecules. The nature of these constituents is not clear at the moment, but may include intramembranous protein particles [15—17],

submembrane (cytoskeleton) structures [18—23] or superficial structures, like villi [23—26]. Whatever the precise origin or nature of what we may call the barrier to entry and lateral movement, the differences we have found point to some types of malignant (and blast) cells having a more 'rigid' membrane that that of a normal, non-dividing, 'resting' cell. In the past, conclusions that a higher 'fluidity' and component mobility were operative in malignant cell membranes have been presented [27—29] but recent studies have questioned the general validity of these assertions [5—9]. The slower spread of F20C through the cell membrane of malignant cells might be due to the enhanced affinity of the probe for protein components of the cell membrane or to a lateral phase separation in which the probe resides in the more highly organized component of the bilayer. Both the precise mechanism of probe entry into the membrane and the detailed reasons for different spreading rates in different cell membranes are promising problems which should be further explored.

Various means have been utilized to study the lateral movement of proteins and lipids in artificial and natural membranes, including fluorescence of modified proteins [30—33], electron spin resonance of spin-labeled phospholipids [14], excimer formation from pyrene [34,35] and quenching of fluorescence by added molecules (anesthetics) [36]. The use of the A_2C-F20C combination permits for the first time a simple, visual way for observing the spread of lipid-like molecules through cell membranes. It is interesting that the apparent diffusion constant for F20C in the presence of membrane mobility agent A_2C within the membrane is $>5 \cdot 10^{-8}$ cm^2/s at 37°C, a value greater than that for phospholipids but not unreasonable in view of the single alkyl chain of F20C and the bilayer "fluidity" promoted by A_2C. The lipophilic character of F20C would suggest residence in and diffusion through the bilayer, but a "hopping" mechanism (a jump into the water layer surrounding the cell, rapid diffusion and return to the bilayer) is not excluded.

Further studies on the modulation of reagent entry and spread in the presence of other agents appear to be practical and should provide further insight into the behavior of cell membranes.

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References

1 Kosower, E.M., Kosower, N.S., Faltin, Z., Diver, A., Saltoun, G. and Frensdorff, A. (1974) Biochim. Biophys. Acta 363, 261—266
2 Kosower, N.S., Kosower, E.M. and Wegman, P. (1975) Biochim. Biophys. Acta 401, 530—533
3 Lustig, S., Pluznik, D.H., Kosower, N.S. and Kosower, E.M. (1975) Biochim. Biophys. Acta 401, 458—467
4 Lustig, S., Kosower, N.S., Kosower, E.M. and Pluznik, D.H. (1977) Proc. Natl. Acad. Sci. U.S. 74, 2880—2884
5 Albrecht-Bhuler, G. and Solomon, F. (1974) Exp. Cell Res. 85, 225—233
6 Gaffney, B.J. (1975) Proc. Natl. Acad. Sci. U.S. 72, 664—668
7 Fuchs, P., Parola, A., Robbins, P.W.and Blout, E.R. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3351—3354
8 Mintz, U. and Sachs, L. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2428—2432

9 Horwitz, A.F., Hatten, M.E. and Burger, M.M. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3115—3119
10 Peavy, D.L., Adler, W.H. and Smith, R.T. (1970) J. Immunol. 105, 1453—1458
11 Dunn, T.B. and Dotter, M.J. (1957) J. Natl. Cancer Inst. 18, 587—601
12 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4457—4461
13 Edidin, M. (1974) Annu. Rev. Biophys. Bioengineer. 3, 179—201
14 Brûlet, P. and McConnell, H.M. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1451—1455
15 Furcht, L.T. and Scott, R.E. (1975) Exp. Cell. Res. 96, 271—282
16 McIntyre, J.A., Gilula, N.B. and Karnovsky, M.J. (1974) J. Cell Biol. 60, 192—203
17 Gilula, N.B., Edgar, R.E. and Rifkin, D.B. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3594—3598
18 Yahara, I. and Edelman, G.M. (1975) Ann. N.Y. Acad. Sci. 253, 455—469
19 Unanue, E.R. and Karnovsky, M.J. (1974) J. Exp. Med. 140, 1207—1220
20 Berlin, R.D., Oliver, J.M., Ukena, T.E. and Yin, H.H. (1974) Nature 247, 45—46
21 Gruenstein, E.R., Rich, A. and Weihing, R.R. (1975) J. Cell Biol. 64, 223—234
22 Oliver, J.M., Albertini, D.F. and Berlin, R.D. (1976) J. Cell Biol. 71, 921—932
23 Loor, F. (1976) Nature 264, 272—273
24 Loor, F. and Hägg, L.B. (1975) Eur. J. Immunol. 5, 854—865
25 Collard, J.G. and Temmink, J.H.M. (1975) J. Cell Sci. 19, 21—32
26 Knutton, S., Sumner, M.C.B. and Pasternak, C.A. (1975) J. Cell Biol. 66, 568—576
27 Shinitsky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603—615
28 Nicolson, G.L. (1973) Nature 243, 218—220
29 Barnett, R.E., Furcht, L.T. and Scott, R.E. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1992—1994
30 Cherry, R.J. (1975) FEBS Lett. 55, 1—7
31 Edidin, M., Zagyansky, Y. and Lardner, T.J. (1976) Science 191, 466—468
32 Schlessinger, J., Koppel, D.E., Axelrod, D., Jacobson, K., Webb, W.W. and Elson, E.L. (1976) Proc Natl. Acad. Sci. U.S. 73, 2409—2413
33 Fernandez, S.M. and Berlin, R.D. (1976) Nature 264, 411—415
34 Soutar, A.K., Pownall, H.J., Hu, A.S. and Smith, L.C. (1974) Biochemistry 13, 2828—2836
35 Vanderkooi, J.M. and Callis, J.B. (1974) Biochemistry 13, 4000—4006
36 Koblin, D.D., Pace, W.D. and Wang, H.H. (1975) Arch. Biochem. Biophys. 171, 176—182
37 Coons, A.H. and Kaplan, M.H. (1950) J. Exp. Med. 91, 1—13
38 Steinbach, G. (1974) Acta Histochem. 50, 19—34
39 Kosower, E.M., Kosower, N.S. and Wegman, P. (1977) Biochim. Biophys. Acta 471, 311—329